THE PENETRATION OF BACTERIA THROUGH CAPILLARY SPACES.

IV. A KINETIC MECHANISM IN INTERFACES.

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PLATES 23 AND 24.

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Before undertaking a more direct study of the physical-chemical factors involved in the penetration of bacteria through the epithelia of the animal body, and as an essential guide in such an investigation, it has seemed desirable to study mechanisms capable of transporting bacteria in and through simpler systems. Certain critical relations of bacterial dimensions and motility to pore size of filters,[1] effects of electric charge and electroendosmotic streaming,[2,3] and bacterial chemotropisms[4] have already been considered. The following notes describe a kinetic mechanism dependent primarily upon interfacial surface tension forces in the boundary between two wholly or partly immiscible fluids. The question whether or not such a mechanism plays a part in actual processes of infection will have to wait upon further study.

TECHNIQUE.

The preparations studied are two-phase liquid films with bacteria suspended, usually in the aqueous phase. Films and bacteria are made visible with a dark-ground illuminator. A small drop of one liquid, usually distilled water, sea water, or Ringer-Tyrode solution, is placed upon a clean slide on the microscope stage. Bacteria, scraped with a platinum loop from an agar slant, are suspended in this

[1] Mudd, S., *J. Bact.*, 1923, viii, 459.

[2] Mudd, S., *Am. J. Physiol.*, 1922–23, lxiii, 429.

[3] Mudd, S., and Mudd, E. B. H., *J. Bact.*, 1924, ix, 151.

[4] Warren, S., and Mudd, S., *J. Bact.*, 1924, ix, 143.

drop of liquid. A clean cover-slip is laid over the suspension.[5] It is desirable that the drop be large enough to give a good film beneath the cover-slip but not so large as to cover the entire under surface of the cover-slip. A drop of the organic liquid—oil, hydrocarbon, alcohol—is caused to run under the cover-slip from one side, meeting the aqueous phase and with it forming a two-phase film between cover-slip and slide. In some organic liquids the bacteria can be suspended first and the water then added. In general, however, this procedure is less satisfactory, because from most organic films thus examined ordinary bacteria are rapidly absorbed on the glass. An alternative method is to place drops of the two liquids to be used at two points on the slide, suspend the bacteria in one of them, and carefully lay a cover-slip over both.

The preparation may then be studied with the dark-field and any desired set of lenses, including the oil immersion. If the organic liquid is too volatile or if it is desired to preserve the preparation for study on subsequent days, the edges of the cover-slip are sealed with a high melting point paraffin. Grübler's 68–72°C. paraffin has been used in our experiments with satisfaction. Paraffin of lower melting point (52°C.) and vaseline have been found to melt in the radiation from the Bausch and Lomb substage illuminator used.

If the preparation is too brilliant for convenient study, the light can, of course, be cut down as desired with a slide-wire rheostat.

OBSERVATIONS.

The boundary line or interface between organic and aqueous phases appears as an intensely luminous line or band, usually with a number of alternate dark and bright interference fringes parallel to, and, typically, on the water side of the interface. The two phases are dark grey or brown or black, according to their optical purity and the illumination. The bacteria appear as golden motes in the interface and water phase and in the organic liquid usually as more silvery particles adherent to the glass. Air bubbles are ordinarily recognizable by their surrounding border of even more dazzling brilliancy than the liquid-liquid interface.

[5] The slides and cover-slips should first be washed with soap and water, then soaked or boiled in dichromate-sulfuric acid cleaning solution, washed, soaked in alcohol, and finally washed in a jet of steam. After the soap and water washing they are handled only with metal forceps. The slides may be kept until used in a clean dry staining dish and the cover-slips in a closed container, preferably on clean filter or lens paper. If necessary they may be carefully wiped with clean lens paper before using.

Observation of such preparations has resolved itself into a study of mechanisms for the concentration of bacteria in the liquid-liquid interface, their transportation, often for considerable distances along the interface, and either their eventual escape from the interface or their agglomeration into solid or semisolid bands in the phase boundary lines. The second phenomenon mentioned, namely movement of bacteria along the interface between organic liquid and water, is more relevant to the general subject of these studies, and will, therefore, be described before considering the mechanism of concentration of bacteria in the interface.

Interfacial Kinetic Mechanism.—The bacteria in the interface were subject to forces which made their behavior distinctively different from that in either water or organic phase. The most characteristic phenomenon observed was a sliding or streaming along the interface. Such movements occurred in films of every composition studied, whether interfacial tension was low or high, and whether the water and organic fluid were miscible or immiscible. The bacteria slid along the bright interfacial line so slowly that their motion could scarcely be detected, or so swiftly that with the high powers of the microscope it could be followed only with difficulty. Individuals and masses were carried along the interface as though in a groove, following the straight or intricately folded boundary line often through several low power fields (each field of diameter 1.24 mm.)

The streaming of microorganisms along the boundary often carried them to a certain point at the margin of the cover-slip. In other places movement was from both directions to a zone in the interface in which the bacteria were crowded together to form a practically solid membrane; subsequently streaming away from this zone in both directions might occur.

Such movements were for the most part independent of movement in the contiguous phases. Often a drift of the bacteria in the water and organic liquid would occur as a result of evaporation or other cause, while the bacteria in the interface a few microns away were streaming in the opposite direction. Or the bacteria in the interface would slide by bacteria in the phases which were not suffering any displacement; or those in the liquids would drift by stationary bacteria in the boundary line. Certain correlations, however, did occur. For

instance, if large bacteria or, especially, bacterial masses were sweeping along the interface, the bacteria nearby were often carried along somewhat less rapidly in the same direction, owing doubtless to the internal friction of the liquid. And when strong currents were produced in the film, for instance by mechanical pressure on the coverslip, these often affected bacteria both in the phases themselves and, in a lesser degree, in the interface.

Certain characteristics of these interfacial displacements could be correlated with the properties of the fluids used. For instance, when interfacial tension was high (as in the case of cyclohexane, tension of water-organic liquid interface (T_{ow}) = 60.6 dynes per cm.) the movements of bacteria in the interface were often jerky and spasmodic, irresistably conveying the impression of particles fixed in a taut elastic medium which yielded locally at times. When interfacial tension was low and the two liquids were to a considerable degree miscible, as in the case of the alcohols (Table I), motion in the interface was more a streaming, often vigorous but always smooth.

Movements of bacteria along the interface occurred, as has been said, in films of all compositions studied. The organic liquids used in this study have been the following neutral and essential oils, hydrocarbons, and alcohols: sperm, sheep's foot, rape-seed, olive and cedar oils, eucalyptol, oils of lavender, thyme, sweet birch, sassafras, and peanut; liquid albolene, liquid petrolatum Squibb, cyclohexane; cyclohexanol, isobutylic and isoamylic alcohols. Caprylic and oleic acids, carbon tetrachloride and ortho-toluidine have shown similar behavior. Distilled water, sea water, and Ringer-Tyrode solution have been used as aqueous phase.

The bacteria used have been the Gram-negative *Bacterium coli communis*, *Vibrio percolans*, and *Erythrobacillus prodigiosus*, and the Gram-positive *Bacillus subtilis* and *Bacillus megatherium*. No significant differences between them as regards the phenomena under discussion have been observed. Under the circumstances of the present experiments the bacteria were non-motile or virtually so. The behavior of bacteria in an actively motile condition and of members of the acid-fast group will be discussed in the following paper.

Similar gliding movements of bacteria along water-air interfaces have also been observed. These interfaces are so dazzlingly bright, however, that they are difficult to study.

Other Movements.—Other movements in the system may now briefly be described. Irregular drifts and currents in the water and organic liquids due to evaporation from the margin of the preparation, pressure, unequal expansion with heating and what not are often much in evidence. When the edge of the cover-slip is sealed with paraffin, these irregular currents in the phases are minimized but usually not wholly eliminated, particularly if air bubbles are contained beneath the cover.

TABLE I.

Interfacial Tension and Solubility of Some of the Organic Liquids Used.

Substance.	Solubility in water per 100 cc.	Interfacial tension (T_{ow}) against water per cm.
	gm.	*dynes*
Cyclohexane.	Insoluble.*	60.60†
Cyclohexanol.	5.67 (11°C.)‡	3.92†
Carbon tetrachloride.	0.80 (20°C.)‡ p. 239.	52.63†
Caprylic acid.	0.25‖	8.217†
Isobutylic alcohol.	Gm. alcohol per 100 gm. at 20°C. ‡ p. 164 Water layer 9 Alcohol layer 84	1.76†
Isoamylic "	Gm. alcohol per 100 gm. at 22°C.§ Water layer 2.61 Alcohol layer 97.36	4.42†
Liquid petrolatum (Squibb).		55.32**

* Hodgman, C. D., and Lange, N. A., Handbook of physics and chemistry, Cleveland, 1922, 180.

† Harkins, W. D., Brown, F. E., and Davies, E. C. H., *J. Am. Chem. Soc.*, 1917, xxxix, 356.

‡ Seidell, A., Solubilities of inorganic and organic compounds, 2nd edition, enlarged and revised, New York, 1919, 280.

§ Seidell, A., Solubilities of inorganic and organic substances, 2nd edition, New York and London, 1911, 11.

‖ Thorpe, E., A dictionary of applied chemistry, London, New York, Bombay, and Calcutta, 1912, 723.

** Harkins, W. D., and Feldman, A., *J. Am. Chem. Soc.*, 1922, xliv, 2665.

The bacteria free in the water undergo active brownian movement. Upon preparation of the film relatively few of the bacteria usually are stuck to the glass. As time goes on, more and more adhere. In the organic liquid such few bacteria as are suspended do or do not undergo brownian movement, according to the viscosity of the medium. In a large majority of the preparations studied, however, the bacteria, if they become suspended in the organic liquid at all very soon adhere to the glass separately or in clumps. A number of times groups of bacteria, lately come into the organic liquid from the water phase, have been observed to clump more closely together, as though drawn by an invisible elastic membrane, and to stick to the glass.

The bacteria in the interface also usually undergo brownian movement unless clumped or stuck to the glass. In some thirty-three experiments in which this point was studied, twenty-three record that brownian movement occurred in the interface. Ten show that it could not be observed there. The movement, however, was usually less free than in the water. The amplitude of the oscillations was less than in the water and the positions of the axes of the bacteria underwent less change than in the water.

As a result of the drifts and currents described, the organic liquid-water boundary line moves about considerably. As it catches, so to speak, on bacterial masses stuck to the glass, the interface sometimes tears the bacteria loose from the glass and sweeps them ahead and on the aqueous side of the advancing boundary line; more often perhaps the interface is itself increased in area and drawn out into rounded projection (interfacial tension high (see Fig. 4)) or finger-like or even very pointed peninsulas (interfacial tension low). The pointed projections are naturally most common in films with very low interfacial tension, the alcohols against water, for instance (see Fig. 2). If the peninsulas were drawn out too long they either retracted, often leaving bacteria individually or in clumps in the organic liquid, or they broke off, leaving a droplet of water containing bacteria in the organic phase (see Fig. 3).

The freedom of brownian movement of bacteria in the organic liquid-water interfaces was less with cyclohexane than with its corresponding alcohol. No brownian movement was noted in four of six experiments with cyclohexane-water interfaces. In the cyclohexanol-

water boundary line very little restraint of the brownian movement of the suspended microorganisms was observed.

The freedom of brownian movement in the interfaces and escape from the interface show interesting correlation. If we designate the fact that bacteria in any given experiment were observed to escape from the interface as + and that none were observed to escape as −, and similarly that brownian movement was observed in the interface as + and that none could be detected as −, in the records of twenty-four experiments, we find the two observations alike in sign in twenty-one and different in sign in only three experiments.

A number of times with cyclohexane-water a long bacterium was observed with one end in the interface and the rest in the water. The part of the microorganism in the water danced about the end in the interface as if about a pivot fixed in a groove, as the whole organism slid along the interface, slowly following its hollows and crests like a minute traveling flail.

Interfacial Trapping Mechanism.—It is characteristic of the preparations under discussion, with the exception of the alcohols and other organic liquids the surface tension of which against water is very low, that bacteria accumulate in the interface in higher concentration than in the liquid phases proper. This accumulation tends to occur more rapidly and to a greater degree in boundaries between liquids of which the interfacial tension is high than in those whose interfacial tension is lower. An hour after preparation of a cyclohexane-water film, for instance, many regions of the interface are usually immobilized by solid or virtually solid membranes of adsorbed bacteria. After a few hours, strong currents may often be caused in such a preparation by pressure on the cover-slip without displacing the interfaces fixed by adsorbed bacteria. With cyclohexanol-water films, on the other hand, the interfaces remain mobile, and streaming of bacteria along them may be seen after days.

The bacteria may reach the interface by brownian movement or with the aid of currents. More effective, however, are movements of the boundary line itself, causing it to encroach on the water phase and overtake the bacterial individuals and masses therein. Careful and patient scrutiny with the oil immersion objective has not shown attraction of bacteria into the interface through microscopically

visible distances. Such perceptible attraction was not to have been expected since the range of action of molecular attraction is of the order of magnitude of 10^{-8} cm. and thus is not capable of microscopic resolution.[6]

When once in the interface, however, the bacteria are trapped there and do not escape, unless work is done upon them. To remain in the interface is so much the rule for suspended bacteria and to leave it the exception that it is perhaps best to enumerate the means by which escape of bacteria from the boundary line has been effected. It is to be remembered, however, that bacteria adsorbed on the glass in the water were engulfed by the organic liquid as it encroached on the aqueous phase in practically all experiments.

Escape from Interface into Organic Liquid.—In thirty experiments there is definite record that suspended bacteria were not seen to cross from the water phase or interface into the organic liquid. In a number of experiments this point was not recorded. In twenty-one experiments bacteria were observed to cross into the organic phase under the special circumstances detailed below.

1. Bacteria, especially when accumulated in masses in the interface, often lagged behind it into the organic liquid as the boundary line gently advanced on the water. This occurred usually when there was reason to believe, because of the composition of the film or the scalloped contour of the interface, that interfacial tension was low. It was noted in eight out of twelve experiments with littoral sea water, which is known to contain surface-active substances, and may have occurred, but was not specially recorded in the others. This lagging behind the interface was, otherwise, noted especially with the alcohols. The following instances are recorded: sea water against oils of lavender, sweet birch and peanut, olive oil, sheep's foot oil, eucalyptol, albolene and *o*-toluidine; distilled water against eucalyptol, isoamylic alcohol, cyclohexanol (three experiments in five), cyclohexane (one in six experiments), $C Cl_4$; Ringer-Tyrode solution against cyclohexanol.

2. When a peninsula of water containing bacteria retracted, bacteria were often left behind in the organic liquid in a little water vacuole or stuck to the glass, and sometimes freely suspended in the oil (see Fig. 3). Sperm oil-distilled water; oil of sassafras-distilled water; $C Cl_4$-distilled water.

3. When suspended clumps of bacteria were overtaken by a rapidly advancing interface they sometimes broke through into the oil: sea water against olive oil and oils of lavender and sweet birch; distilled water-sperm oil.

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[6] Lewis, W. C. McC., A system of physical chemistry, London, New York, Bombay, Calcutta, and Madras, 2nd edition, 1918, i, 10.

4. When bacteria were strongly swept along the interface into a certain region until masses were formed and these became too large, certain bacteria were forced out of the interface into the organic phase; eucalyptol-sea water, peanut oil-sea water; cyclohexanol-distilled water; cyclohexane-distilled water.

5. Occasionally bacteria in active brownian movement in the interface danced out into the organic phase: eucalyptol-sea water; cyclohexanol-distilled water; C Cl_4-distilled water.

6. When bacteria were rounding a sharp curve in the boundary line at high speed, they were thought in several instances to be flung out by centrifugal force into the oil; sperm oil-distilled water (two experiments).

Escape from Interface into Water.—There are many circumstances also in which bacteria escape from the interface into the aqueous phase. The following examples may be cited.

1. When bacteria had been carried into one region of the interface until large numbers accumulated there, some were forced out of the interface into the water: sea water-eucalyptol.

2. Bacteria in brownian movement in the interface danced out into the water: sea water-oil of lavender, sea water-oil of thyme, distilled water-cyclohexanol (two experiments), distilled water-caprylic acid.

3. When bacteria were swept at high speed down the interface, they occasionally struck masses of fixed bacteria with sufficient force to be bounced out of the interface into the water: distilled water-cyclohexanol.

4. Bacteria rounded a sharp bend in the interface so fast as to be thrown out into the water: sperm oil-distilled water; cyclohexanol-distilled water.

5. When an oil phase not in contact with water ran against the aqueous phase, some bacteria were knocked out of the air-water interface into the water: eucalyptol-distilled water.

In examining the accompanying plates it should be remembered that the films have appreciable depth, and that the organic liquid, water, and probably the interface have different indices of refraction. It is not possible, therefore, to bring all parts of the preparation into a single focal plane. A wealth of detail and the fine definition which can only be brought out by constant manipulation of the fine adjustment are therefore necessarily lost in photography.

The preparations taken through low power lenses—× 165—were sealed and allowed to stand for a few hours, after which they were still enough to photograph.

For high power views, Mr. Louis Schmidt took moving picture films and enlarged individual pictures. The film from which Fig. 5 is taken very successfully showed the streaming movements in the interface and the brownian movement of bacteria in the water phase. In the enlargement, bacteria the excursions of which in the focal plane were large, *i.e.* those moving in the interface and several in the water, appear as bright streaks rather than points. The bright specks visible in the interface at *p* and *q*, Fig. 5, were bacteria stuck to the glass.

Interpretation.

It is clear from inspection of the bacteria in the aqueous-organic liquid interfaces of 60 odd experiments that work must be expended upon the bacteria to remove them from the interface when once there. It seems safe to deduce from the Gibbs-Thomson principle therefore that the presence of the bacteria in the interface lowers the free surface energy of the interface. This conclusion harmonizes with the observation that the escape of the bacteria from the interface seems to be more difficult when the interfacial tension is high than when it is low, *e.g.* bacteria are more effectively trapped in cyclohexane-water than in cyclohexanol-water interface.

The movement of bacteria along the interface has occurred in films of all composition studied, including those in which the water and organic liquid are not appreciably miscible, *e.g.* cyclohexane, mineral oil. This streaming in the interface occurs whether or not there is any drift of the particles in the contiguous water and organic phases and often in opposite direction to displacements of the bacteria in the phases if such are occurring. Similarly drift of the water and oil may occur past stationary bacteria in interfaces. Moreover, displacements of bacteria in interfaces of high surface tension are often distinguished by their jerky, tense character. From these facts we may conclude that local changes in surface tension caused, for instance, by local concentration of surface-active substances or uneven heating of the interface, constitute one driving mechanism for the bacteria in their movement along the interface.

On the other hand, streaming of the bacteria is quite as vigorous in interfaces between fluids whose interfacial tension is low but whose mutual solubility is considerable (*e.g.* isoamylic, isobutylic alcohols, and cyclohexanol against water) as in interfaces between liquids of high interfacial energy which are immiscible. In such preparations of miscible liquids, moreover, microscopic whirlpools and currents in the phases near the interface are likely to occur. We conclude from these facts that minute currents along and across the interface, due to mixing of the two liquids, constitute a driving mechanism for the bacteria in addition to, although not wholly separable from, changes in surface tension.

A more detailed analysis of the phenomena here recorded in terms of the interfacial surface tension relations will be given in the paper immediately following.

DISCUSSION.

Quincke[7] in 1879 observed a drop of neutral oil containing a little fatty acid and suspended in alkali solution. The fatty acid and alkali met at the surface of the oil drop, formed soap and this, lowering surface tension locally, was spread by the tension of the interface over the surface of the drop. Some of the contiguous water was drawn along by the spreading soap film and other water moved into its place, thus giving rise to currents in the solution. Loeb and others have applied such observations to the explanation of certain intracellular phenomena.[8,9] The movements of bacteria along the interfaces described in the present communication are evidently similar phenomena to those of spreading described by Quincke. Movements of bacteria along lipoid-water interfaces have been predicted by Wright on theoretical grounds and urged

[7] Quincke, G., *Arch. ges. Physiol.*, 1879, xix, 129; *Sitzungsber. berl. Akad. Wissensch.*, 1888, 791.

[8] Loeb, J., The dynamics of living matter, New York and London, 1906, 55, 65.

[9] Ewart, A. J., On the physics and physiology of protoplasmic streaming in plants, Oxford, 1903, 112.

by him as of capital importance in the initiation of infection.[10] We should prefer to reserve judgment.

SUMMARY.

The dark-field microscope may be used to observe directly the characteristics of composite films. The liquid phases, one or both of them containing suspended solid particles as test objects (in these experiments bacteria were used), are spread between slide and coverglass and examined with any desired lenses. The liquid-liquid interfaces appear as bright lines and the solid particles as shining motes.

An interfacial kinetic mechanism has been observed in films of all composition studied. The bacteria are transported along the phase boundary lines in a striking and characteristic manner and quite independently of movements in the adjoining organic or aqueous phases. These movements in the interface are interpreted as essentially due, according to the composition of the films, to local inequalities in interfacial surface tension, or to minute currents from mixing of the two phases across and along their boundary line, or to both forces acting together.

The bacteria (non-motile in these experiments) reached the interface by brownian movement or currents or shifts in the position of the boundary line. Once in the interface they tended to remain, and accumulated there, in instances where the liquid-liquid interfacial tension was high at least, in higher concentration than in the contiguous phases. Bacteria could, however, escape from the interface in a variety of ways detailed above.

With liquids which differ markedly in interfacial tension and miscibility with water, these properties may be correlated with the characteristics of the preparation. With cyclohexane-water films, for instance, (immiscible, interfacial tension high), the boundary was less readily drawn out into projections, the interfacial trapping mechanism was more efficient, and brownian movement of bacteria

[10] Wright, J., *New York Med. J.*, 1906, lxxxiii, 17; lxxxiii, 117; lxxxiv, 1161; 1907, lxxxv, 289, 435, 537, 627, 769; 1910, xcii 749; 1911, xciii, 257, 354, 662, 764.

in the interface was less free than with cyclohexanol-water films (miscible, interfacial tension low).

Analysis of the mechanism of the phenomena herein described will be given in the paper following.

Some of the oil samples used in these experiments were given us by Dr. W. T. Bovie of the Harvard Biophysics Laboratories, whom we take this occasion to thank.

EXPLANATION OF PLATES.

The fine definition and wealth of detail which can be brought to the observer's eye, for reasons indicated above are necessarily lost in photography of these preparations.

PLATE 23.

FIG. 1. Cyclohexane-distilled water-*Bacterium coli*. × 138. Preparation photographed within 3 hours. The adsorbed bacteria form a semisolid band in the interface, appearing in the figure as a bright, heavily stippled line. Note the straight interface. Bacteria form heavy suspension in water; are almost absent from organic phase.

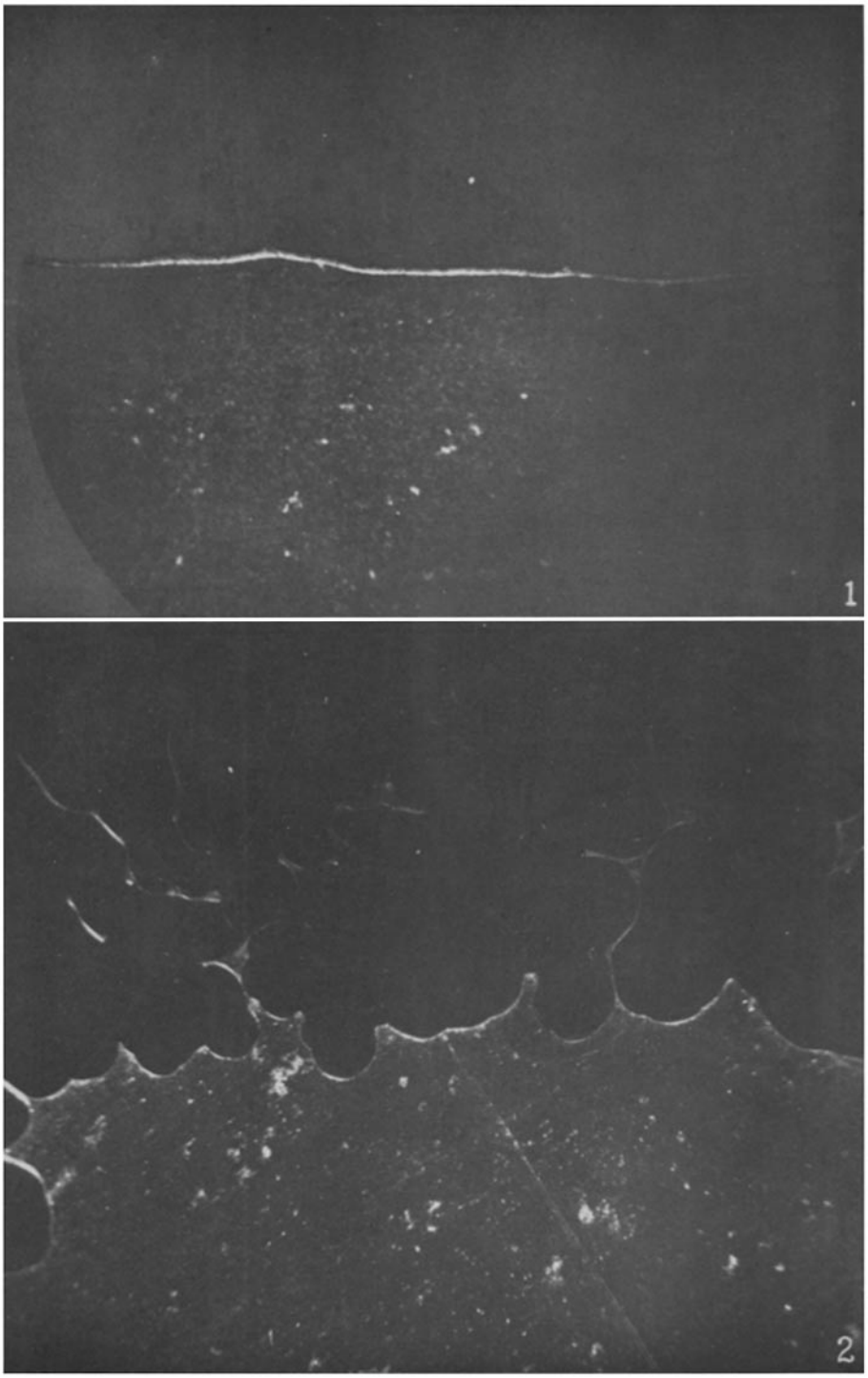
FIG. 2. Cyclohexanol-distilled water-*Bacterium coli*. × 138. Preparation photographed after about 3 hours. Sharply scalloped interfacial line. Less adsorption of bacteria in interface than with cyclohexane.

PLATE 24.

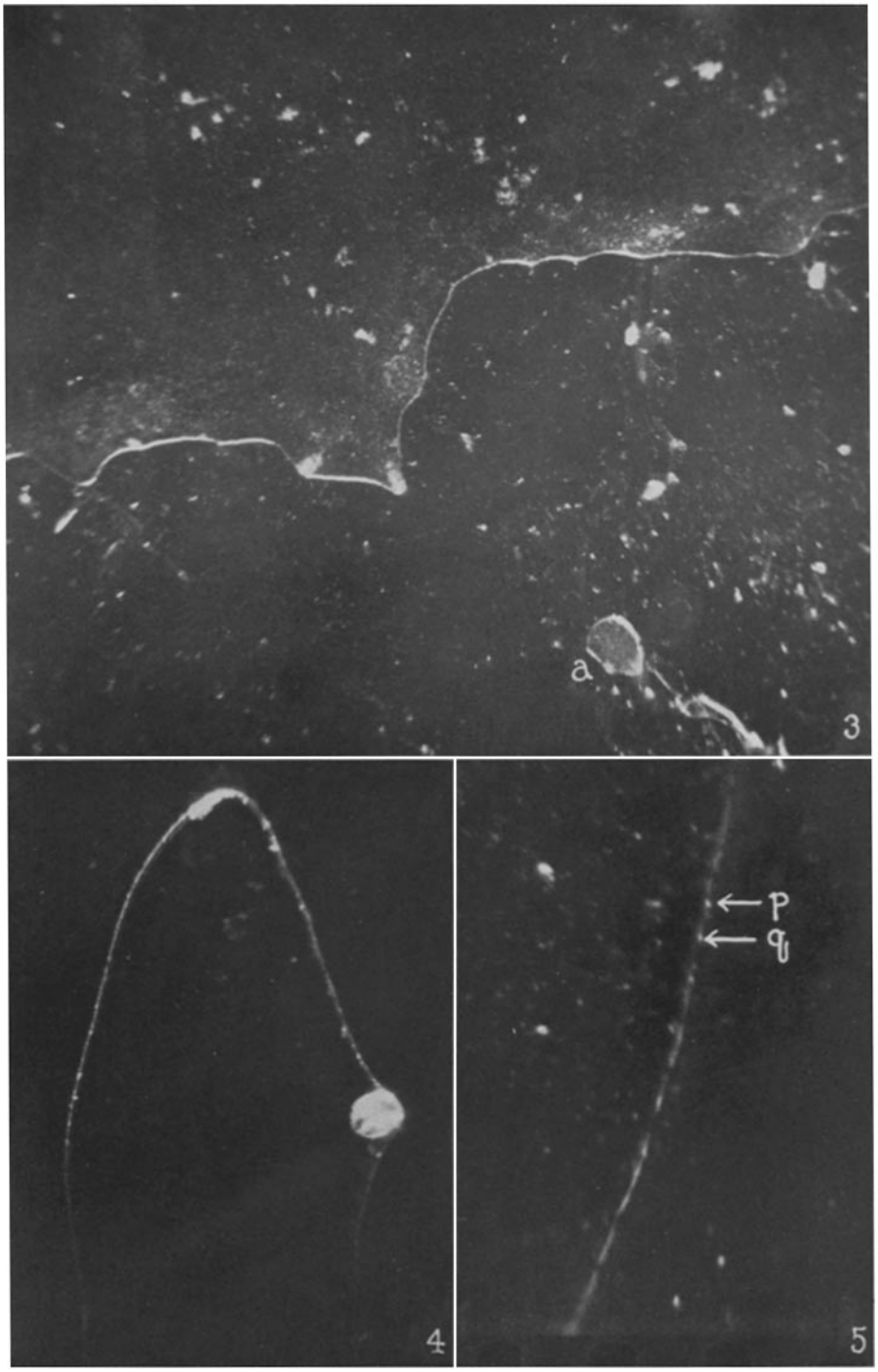
FIG. 3. Sperm oil-distilled water-*Bacterium coli*. × 138. Preparation photographed after about 24 hours. A vacuole of water containing microorganisms is shown enclosed in the oil at *a*. Bacterial individuals and clumps appear as bright specks.

FIG. 4. Cyclohexane-distilled water-*Bacterium coli*. × 292. Fresh preparation. Bacteria in interface give beaded appearance. Plate underexposed for bacteria in hydrocarbon and water phases.

FIG. 5. Cyclohexane-distilled water-*Bacterium coli*. × 625. Fresh preparation. Enlargement from moving picture film. Bacteria moving along interface appear as streaks, as do several undergoing brownian movement in water. Bright specks at *p* and *q* are bacteria in interface stuck to glass.



(Mudd and Mudd: Penetration of bacteria IV.)



(Mudd and Mudd: Penetration of bacteria. IV.)